RESEARCH ARTICLE

Molecular Reproduction & Development 77:978–989 (2010)

Characteristics of Peri-Implantation Porcine Concepti Population and Maternal Milieu Influence the Transcriptome Profile

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SUMMARY

Asynchrony of trophectoderm elongation, gestational days 11–12, is evident in porcine concepti, and rapid progression through this phase has been associated with conceptus competency. The goal of the current study was to determine the extent of transcriptomic responses of concepti to developmental delay and their physiological implications. Gestational day 11 concepti with the same morphology, ovoid and 7–8 mm, were isolated and designated as control or developmentally delayed if collected from a homogenous ovoid conceptus population or heterogeneous conceptus population (ovoid to filamentous), respectively. Total RNA prepared from four distinct control and four distinct developmentally delayed concepti, was analyzed using an Agilent high-density custom porcine microarray. Two hundred nine transcripts were found differentially expressed between normal and developmentally delayed concepti. Functional analysis of these genes indicated that a significant number of the genes regulate signal transduction/transcription, organismal development, metabolism, and cell adhesion and can be modulated by transforming growth factor β1 (TGFβ1). Ten genes were selected for real-time PCR validation of differential expression based on a known role in steroid synthesis, endometrium receptivity, and modulation of trophoblast differentiation/growth or interaction with TGFβ1. As in the microarray, all except one, achaete–scute complex homolog 2, were preferentially upregulated in delayed concepti. Overall, findings suggested that despite similar morphology, the transcriptome of developmentally delayed concepti is distinct from control counterparts. Also highlighted were ways by which the conceptus' microenvironment might be affected and developmental factors that may be of interest to interrogate further to determine if, and how, they affect embryo competency/elongation.

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Supported by USDA ARS CRIS project no.1265-31000-094.

Mol. Reprod. Dev. 77: 978–989, 2010. Published 2010 Wiley-Liss, Inc.†

Published online 15 October 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/mrd.21253

Received 10 May 2010; Accepted 20 September 2010

INTRODUCTION

Ungulate concepti have an extended peri-implantation period during which time the extra-embryonic membranes expand, that is, elongate (Geisert et al., 1982; Stroband and Van der Lende, 1990; Geisert and Malayer, 2000). In swine, elongation of the extra-embryonic membranes provides an

Additional Supporting Information may be found in the online version of this article.

Abbreviations: BP, biological processes; CC, cellular component; CD, control ovoid; DD, developmentally delayed ovoid; E2, estrogen; ED, embryonic disk; FIL, filamentous; gd, gestational days; GO, gene ontology; ICM, inner cell mass; IPA, ingenuity pathway analysis; PC, principal components; QC, quality control.

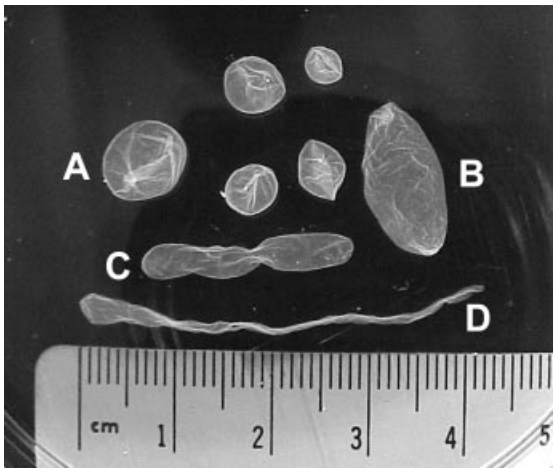

Figure 1. Morphology of elongating embryos. Depiction of the heterogeneity of morphology porcine concepti flushed between gd11 and 12. (**A**) Ovoid, (**B**) early tubular, (**C**) intermediate tubular, and (**D**) transitioning tubular/filamentous.

increased placental surface area to enable maternal–conceptus cross-talk and nutrient exchange that is essential for survival of the conceptus (Stroband and Van der Lende, 1990). The peri-implantation period gestational days (gd) 11–12 defines the early, rapid elongation phase and is a very critical time of development as conceptus loss can approach 18% (Bennett and Leymaster, 1989). Subsequent to this early phase of elongation, attachment of the conceptus to the endometrium begins on gd13, concomitant with continued elongation of the extra-embryonic membranes and is complete by gd18 (Geisert and Malayer, 2000). Successful implantation is dependent upon multifaceted interactions between the maternal and embryonic compartments.

The result of the elongation process is a dramatic change in the gross morphology of the blastocyst as the ovoid conceptus of ~4–5 mm, in length and width, transitions to an intermediate tubular conceptus of a few cm to a long thin filament >10 cm with a diameter of <1 mm, in about 4 hr (Fig. 1) (Geisert et al., 1982; Stroband and Van der Lende, 1990; Geisert and Malayer, 2000). Concurrent with extra-embryonic membrane elongation are more subtle, but, important microscopic changes within the embryonic disk (ED), that is, the embryo proper, namely gastrulation (Marrable, 1971; Blomberg et al. 2006). Synchrony between extra-embryonic membranes and gastrulation stages, based on their gross morphology, is not apparent; however, advancement of the ED to the primitive streak stages seems to be restricted to the filamentous stage (Blomberg et al. 2006). Thus, the gastrulation stage of the ED (Blomberg et al., 2006) or the expression of developmental markers, such as brachyury or POU class 5 homeobox 1 (OCT3/4), varies between embryos exhibiting the same extra-embryonic membrane morphology irrespective of their derivation, that is, a homogenous or heterogeneous population (unpublished work).

Equally important for conceptus implantation and survival is an apposite uterine endocrine milieu along with steroid hormones, growth factors, and cytokines that promote the required gestational alterations (Blair et al., 1991; Geisert et al., 1991). For example, the precise production and secretion of estrogen (E2) by the conceptus, during the peri-implantation period encompassing gd11–12, is important for the maternal recognition of pregnancy and embryo's viability (Perry et al., 1976; Geisert et al., 1982, 1990; Niemann and Elsaesser, 1986; Geisert and Yelich, 1997). The E2 level, otherwise negligible in the uterine luminal fluid of non-pregnant gilts, increases almost seven-fold between the ovoid to filamentous conceptus stage as a consequence of increased steroid synthesis by the conceptus (Geisert et al., 1982). The hormone most likely has (1) a paracrine effect through its action on the endometrium to modulate prostaglandins towards a pro-pregnancy environment and to release mitogenic factors that promote growth of cells within the conceptus trophectoderm (Ka et al., 2001) and (2) an autocrine effect through the potential modulation of trophoblast differentiation via the E2 receptors (Kowalski et al., 2002; Rama et al., 2004). The importance of both a synchronized uterine compartment and the presence of essential developmental factors has been demonstrated by the onset of conceptus loss or abnormal development when E2 levels are increased above normal levels or upon exposure of concepti to a more advanced uterine environment during peri-implantation (Pope et al., 1986; Wilde et al., 1988; Blair et al., 1991; Geisert et al., 1991).

Rapid progression through elongation has been hypothesized to be important for conceptus competency (Pope et al., 1982, 1986; Xie et al., 1990). Therefore, the presence of ovoid concepti in uterine flushings consisting primarily of advanced tubular early-stage filamentous concepti, has raised the question of whether or not the developmentally delayed ovoid (DD) concepti are less developmentally competent, that is, have physiological changes occurred in the DD conceptus that would hinder its development potential compared to an ovoid conceptus from a homogeneous population (controls, CD). Or, considering ovulation can occur over a period of ~4–8 hr (Xie et al., 1990) and elongation is very rapid, the apparent lag in elongation could be merely a result of ''ovulation timing'' rather than developmental competence. ''Ovulation timing'' studies by Xie et al. have demonstrated that oocytes ovulated later give rise to less developed preimplantation embryos. Thus, lagging embryos have a disadvantage for further development if most of the endometrium has been occupied by the concepti that elongated early and more rapidly. In addition to uterine space, embryo transfers (Pope et al., 1982, 1986) suggest that asynchrony between the developmental stage of the embryo and endometrium are also important because a less mature embryo placed in a more advanced uterine environment produces a less advanced gd12 peri-implantation blastocyst than its more mature transferred cohorts.

Despite our knowledge of factors and some potential mechanisms that are differentially regulated between different stages of elongation, an in-depth understanding of the actual mechanisms driving or inhibiting extra-embryonic membrane expansion and conceptus survival is still lacking

(Conley et al., 1992; Yelich et al., 1997; Blomberg et al., 2005, 2006; Degrelle et al., 2009; Ross et al., 2009). To determine whether morphology or uterine milieu might be the primary influence on the biological status of the conceptus, the transcriptome of morphologically identical ovoid concepti from homogenous (CD) and heterogeneous (DD) conceptus populations was examined in-depth. Furthermore, the hope was that if different, the change may shed light on factors that could potentially regulate important physiological processes such as elongation. This could be important to pinpoint putative biomarkers of embryo development potential for additional studies.

RESULTS

Global transcriptome profiling of CD and DD ovoid concepti obtained on gd11 was performed utilizing the custom E-array platform from Agilent technologies. The E-array probes were designed from the available mRNA sequence information present in the NCBI Pig Unigene database. A total of 39,908 unique 60-mer oligonucleotide probes were identified but only 39,513 met Agilent's criteria for use as a probe and were used to populate the array. Considering that some of the probes originated from EST sequences or unknown sequences lacking annotation, there was the potential for redundancy of gene targets.

Following array hybridization and scanning, the raw intensity values, that is, the gProcessedSignal (Cy5) and rProcessedSignal (Cy3) values, of independent DD and CD concepti obtained from the Agilent microarray scanner were utilized for quality control (QC) analysis of the data with JMP Genomics software (SAS). The log2 transformed LS means were used to examine array reproducibility and correlation and expression clustering patterns for QC of the microarrays. The correlation coefficient analysis of arrays labeled with Cy3 or Cy5 yielded a coefficient of determination (r^2) between 0.97 and 0.99 for all of the arrays except DD3, which was a little lower, $r^2 = 0.97–0.96$. Supplementary Table 1 shows the comparison and results with the Cy5-labeled arrays; the Cy3-labeled arrays were almost identical (not shown). Cluster analysis with the Cy3 or Cy5 labeled cRNA demonstrated that overall, the expression profile of individual CD and DD ovoid concepti clustered with the distinct conceptus population from which they were derived. The principal components 3D (PC) scatterplot showing a clear separation of CD from DD concepti is presented in Figure 2; though not shown, the hierarchical clustering heat map and dendrogram depicted the same outcome. Overall, the PC analysis indicated that the primary driving force of PC1 was the dye label (Cy3 or Cy5) whereas; PC2 was influenced by the treatment, that is, CD or DD. Additionally, the array hybridized with Cy3-labeled cRNA from ovoid conceptus DD3 clustered with the Cy5-labeled DD samples; however, Cy3 and Cy5-labeled DD populations were distinct from the CD.

A total of 209 transcripts were found to be differentially expressed between CD and DD concepti independent of the Cy fluorophores with which they were labeled; 118 transcripts up-regulated and 91 down-regulated. Gene annotation was obtained for 170 of the transcripts and 6 of the transcripts [CDC42 effector protein (Rho GTPase binding) 4 (CDC42EP4), glycerol kinase (GK), inositol polyphosphate-5-phosphatase A3 (INPP5A3), lipin 1 (LPIN1), Sec24-related protein D2 (SEC24D2), synaptopodin 2 (SYNPO2)] were redundant, that is, represented by more than one probe. The duplicate probes exhibited differential expression in the same direction and all but two gave similar fold values. Both CDC42EP42 and SYNPO2 had one probe with a value two times greater than the other (Table 1); whether or not this was due to the detection of multiple variants with one probe but not the other, remains unanswered.

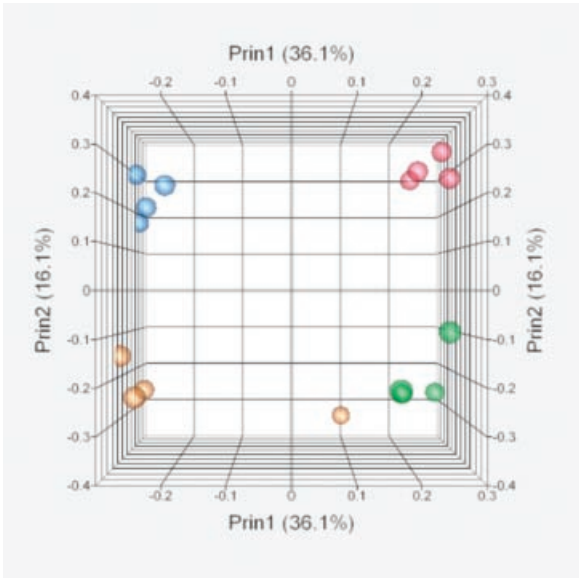

Figure 2. 3D scatterplot of microarrays (JMP Genomics). JMP Genomics software was utilized for quality control analysis of individual microarrays, and to visualize the relationship between the different treatment groups, that is, DD concepti versus CD concepti, and the impact of labeling with different dyes. Blue and red, CD conceptus RNA labeled with Cy3 and Cy5, respectively; yellow and green, DD conceptus RNA labeled with Cy3 and Cy5, respectively. Each colored circle represents a distinct conceptus.

Gene ontology (GO) characterization using GeneCodis 2.0 demonstrated that 22 distinct transcripts could be mapped within GO process whereas 74 mapped within GO function (Table 1). The most notable GO processes and function subsets were multi-cellular organismal development and protein binding, respectively. The arrows in the table pointing up or down are indicative of up- and down-regulation of the specific transcript in the DD conceptus. It is apparent that most of the annotated transcripts mapped to organismal development and lipid metabolism processes are up-regulated in the DD conceptus. Approximately half the transcripts encoding proteins that mapped to protein and nucleotide binding functions are down-regulated and half were up-regulated in DD concepti; transcripts associated with transferase activity and calcium binding were preferentially up-regulated in the DD conceptus. Potential pathways and network gene interactions were identified by ingenuity pathway analysis (IPA). Of the 170 annotated transcripts,

TABLE 1. Gene Ontology (GeneCodis)

Categorization	Gene represented by transcript
GO process	
Lipid metabolism	**SULT4A1** (↑2.24), ALDH3B2 (↓7.69), **LDLR** (↑2.38), LRP2 (↓3.57), **CYP11A1** (↑2.04), **LPIN1** (↑3.32/4.04), **HSD11B1** (↑3.15), **STAR** (↑7.10)
Multicellular organismal development	*SFRP1* (↓5.88), **DPPA5** (↑5.53), **GCNT2** (↑2.65), **SLIT2** (↑1.72), *RYBP* (↓1.56), ***PITX1*** (↑4.95), **NODAL** (↑2.49), ASCL2 (↓2.86), **PLXNB1** (↑1.81), **DISP1** (↑1.70)
Anatomical structure and morphogenesis	*SFRP1* (↓5.88), **PHLDA3** (↑3.16), ***PITX 1***(↑4.95)
Carbohydrate metabolism	GK (↓3.63/2.95/2.29), **ST3GAL5** (↑2.42), **SLC2A3** (↑1.82), PDK3 (↓2.70)
GO function	
Protein binding	**ZYX** (↑3.22), SFRP1 (↓5.88), FCER1G (↓2.13), ***CLSTN3***(↑1.39), BLNK (↓4.55), TRIM11 (↓2.27), PPFIA3 (↓1.37), **CSRP1** (↑1.88), ***TGM1*** (↑3.88), ***CDH6*** (↑2.23), *THRAP3* (↓2.38), **WWP2** (↑2.09), SNX6 (↓2.94), AP1S2 (↓2.86), BTRC (↓2.94), KCNQ1 (↓3.70), SYNPO2 (↓12.50/5.26), **SHF** (↑4.64), *LRP2* (↓3.57), *RAB3D* (↓1.25), *CACNB4* (↓4.55), ***PI4KA*** (↑1.96), *DHX38* (↓1.32), **CDC42EP4** (↑3.27/7.27), ATP1B3 (↓1.61), FANCD2 (↓2.50), **DSCR1** (↑8.14), PTGES2 (↓1.67), **INTS5** (↑2.09), ***SNF1LK 2***(↑1.21), GPR125 (↓5.56), *MAPK9* (↓1.67), **LIN7B** (↑1.76), **MAGOH** (↑1.68), **STAR** (↑7.10), MAGI1 (↓1.96), **ATXN7** (↑1.66), SGOL1 (↓1.96), *RYBP* (↓1.56), NRD1 (↓3.33), CCS (↑5.14), **PHLDA3** (↑3.16), **PLXNB1** (↑1.81), ***CRY2*** (↑1.70), CTPS (↓2.00), TLR1 (↓2.04), ***WNK1***(↑1.9), SEC24D2 (↓2.50/2.27), *ATP1A1* (↓1.61)
Nucleotide binding	GLUD1 (↓2.56), *THRAP3* (↓2.38), ***GNE*** (↑3.08), *BTK* (↓7.14), **RAB15** (↑1.66), ARL5A (↓1.25), *RAB3D* (↓1.25), **RND3** (↑2.67), *DHX38* (↓1.32), ***SNF1LK2***(↑1.21), ABCA1 (↓2.33), AARSD1 (↓1.59), *MAPK9* (↓1.67), *GK* (↓3.63/2.95/2.29), ***SPHK1*** (↑4.33), ***TAOK2*** (↑1.64), **CHD7** (↑2.12), ***CRY2*** (↑1.70), **CHTF18** (↑5.53), ***WNK1*** (↑1.9), *ATP1A1* (↓1.61)
Transferase activity	*GNPTAB* (↓2.17), ***TGM1*** (↑3.88), ***GNE*** (↑3.08), **SULT4A1** (↑2.24), *BTK* (↓7.14), ***PI4KA*** (↑1.96), ***SNF1LK2*** (↑1.21), *MAPK9* (↓1.67), *GK* (↓3.63/2.95/2.29), ***SPHK1*** (↑4.33), ***TAOK2*** (↑1.64), **LPGAT1** (↑6.78), PDK3 (↓2.70), ***WNK1*** (↑1.9)
Calcium binding	*GNPTAB* (↓2.17), ***CLSTN3*** (↑1.39), **EFCBP2** (↑6.92), ***TGM1*** (↑3.88), ***CDH6*** (↑2.23), **LDLR** (↑2.38), *LRP2* (↓3.57), *CACNB4* (↓4.55), **SLIT2** (↑1.72), **S100A16** (↑1.87)

Gene names in: regular font—up-regulated in CD; bold font—up-regulated in DD; italicized—duplicated in another GO sub-process or sub-function.

72 were down-regulated and 98 were up-regulated in lagging concepti. Of those, 168 genes mapped into either a signaling pathway and/or a gene interaction network. The most significant canonical signaling pathways identified by IPA were transforming growth factor β (TGFβ) subfamily members and Fc fragment of IgG, low affinity IIb, receptor (FCGR2B; aka, CD32); however, they were not highly represented. Components of the FCGR2B pathway, for which a transcript was identified, were B-cell linker protein, B-cell progenitor kinase, and c-jun N-terminal kinase. All three factors reside downstream of the FCGR2B, and their mRNA was preferentially down-regulated in DD concepti. In contrast, the TGFβ family members identified, TGFβ1, TGFβ3, and *nodal homolog* (NODAL), were all up-regulated in DD concepti compared to CD concepti. A look at gene networks indicated that in the most statistically significant network composed of 35 focus genes, TGFβ1 was the most prominent upstream factor (Fig. 3). Within that network, the transcript for 25 of the 35 focus genes were found in the porcine conceptus; 17 were up-regulated in the DD conceptus and 8 were down-regulated. As a whole, the factors have been associated with the regulation of molecular transport, cell nutrition and cellular function/maintenance; all potential processes that are important for promoting conceptus survival. An evaluation of both Figure 3 and Table 1 indicated that TGFβ1 was upstream of factors categorized in GO that modulate lipid metabolism, including steroid synthesis [steroidogenic acute regulatory protein (STAR), 11-β hydroxysteroid dehydrogenase 1 (HSD11B1), and cytochrome $p45_{\text{side-chain cleavage}}$ protein (CYP11A1); highlighted by blue asterisks, Fig. 3], carbohydrate metabolism (GK), and organismal development/morphogenesis [rat Y-box binding protein (RYB) and secreted frizzled-related protein 1 (SFRP1)].

To validate the results of the microarray, 10 factors known to be involved in conceptus/trophectoderm development were chosen for confirmation of their differential expression by real-time PCR. These included TGFβ family members/associated proteins (TGFβ1, TGFβ3, and NODAL), steroidogenic/steroid activity regulating factors [STAR (longest form, Blomberg and Zuelke, 2005), CYP11A1, and mesoderm induction early response 1 homolog (MIER1)], imprinted placental growth/development genes [achaete–scute complex homolog 2 (ASCL2) and H19 imprinted maternally expressed transcript (H19)], a bioavailability regulator of the imprinted gene insulin-like growth factor 2 (insulin-like growth factor binding protein 3, IGFBP3) and an immune modulator (interleukin 1β, IL1β). The microarray analysis indicated beta actin (ACTB) was constitutively expressed between CD and DD embryos, thus ACTB was evaluated by real-time PCR and statistical analysis of the ACTB threshold cycle (C_t) mean ± standard error of the mean (SE; Blomberg et al., 2005). Analyses of the ACTB C_t mean for each embryo population demonstrated the transcript level was similar between CD (18.626 ± 0.168) and DD (18.568 ± 0.156) embryos, thus ACTB was used as the endogenous control.

The mRNA for IL1β was not detected as being different between CD and DD by microarray, but it is known to be differentially regulated during elongation. The expression level of the genes was relative to ACTB; TGFβ1, TGFβ3, NODAL, IGFBP3, STAR, CYP11A1, H19, and MIER were

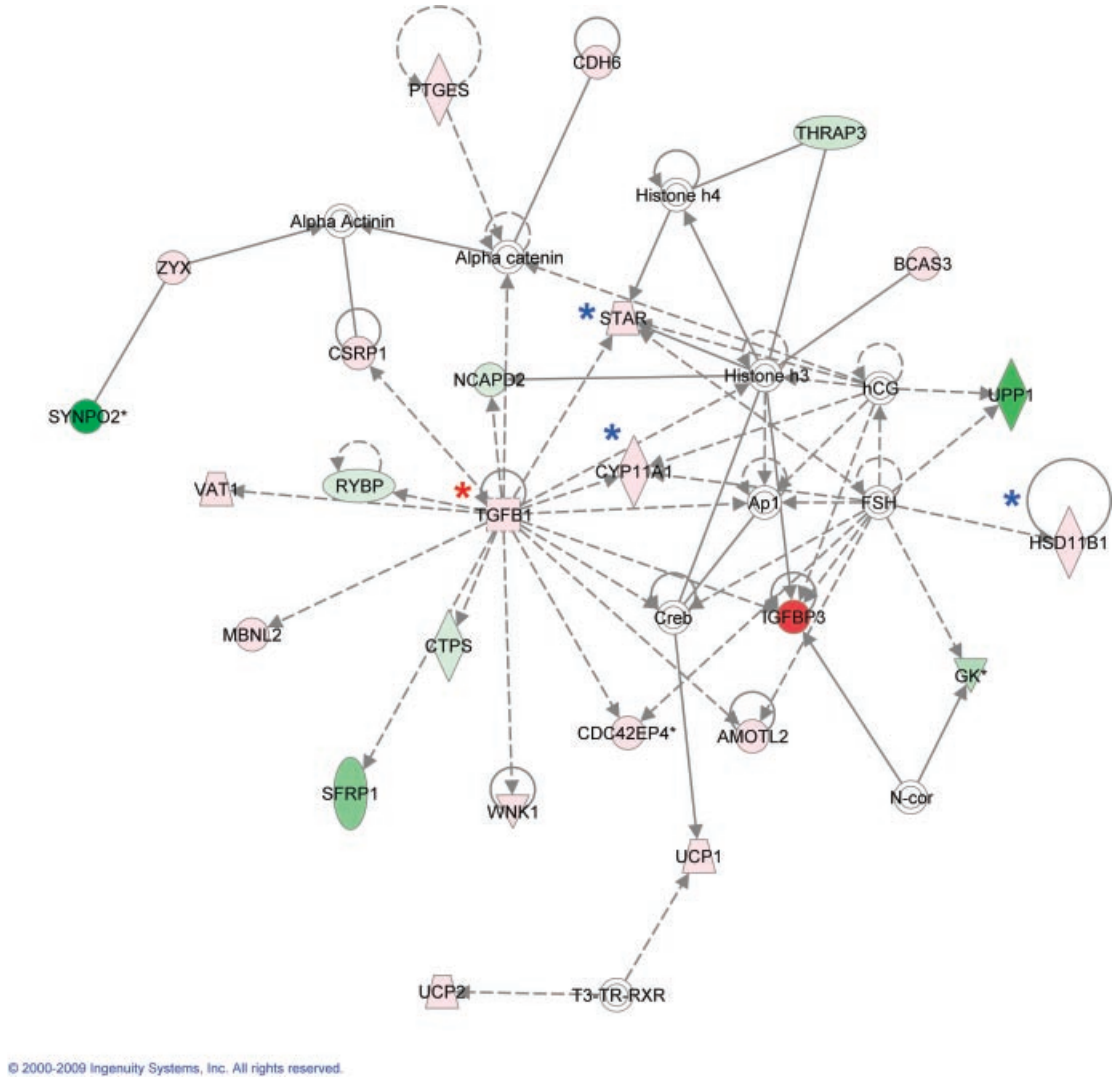

Figure 3. Network analysis by IPA. Transforming growth factor β1 was central to the most significant network identified by IPA. A total of 25 of the 35 network focus genes were detected in the porcine conceptus; 17 (pink to red) of which exhibited up-regulation in the DD conceptus and 8 (green) that were down-regulated in the DD conceptus. The darker the color, the greater the fold difference between DD and CD concepti. The dashed and solid lines emanating from a specific factor towards another is indicative of known indirect and direct interaction between those proteins. TGFβ1 (red asterisk) was central to the network; factors potentially downstream of TGFβ1 involved in steroid synthesis are highlighted with a blue asterisk. Full gene names can be found in supplementary Table 3.

more highly expressed ($P \leq 0.05$) in the DD concepti than CD concepti (Fig. 4A,B; Table 2). The largest differences were 21- and 13-fold increases of the IGFBP3 and TGFβ3 transcripts, respectively, in the DD concepti. In contrast, the level of ASCL2 mRNA was suppressed ($P \leq 0.05$) in DD concepti; the transcript level in the CD was two-fold greater.

Considering DD concepti are present in the uterine tract with more advanced concepti, a comparison of the gene expression between the more advanced gd12 filamentous concepti and the DD concepti was also determined. The transcript level of TGFβ1, TGFβ3, NODAL, IGFBP3, and MIER was not different between DD and filamentous concepti (Table 2) but, ASCL2, IL1β, and H19 mRNA expression was 100%, 60%, and 74% greater ($P \leq 0.05$), respectively, in the DD conceptus than filamentous conceptus. A comparison of the expression level for select transcripts between CD and filamentous concepti indicated the expression directionality mimicked that found between the CD and DD concepti comparison (Table 2). However, in the DD conceptus STAR and CYP11A1 mRNA levels were at intermediate levels between the CD and filamentous concepti, and significantly different from those stages ($P < 0.05$; Table 2); STAR was seven-fold greater in the DD than CD conceptus and two-fold less in the DD than filamentous conceptus, whereas, CYP11A1 was two-fold higher in the DD than CD and 1.7-fold lower in the DD than filamentous conceptus. Thus, significant differences were found between the DD concepti and the other two conceptus populations.

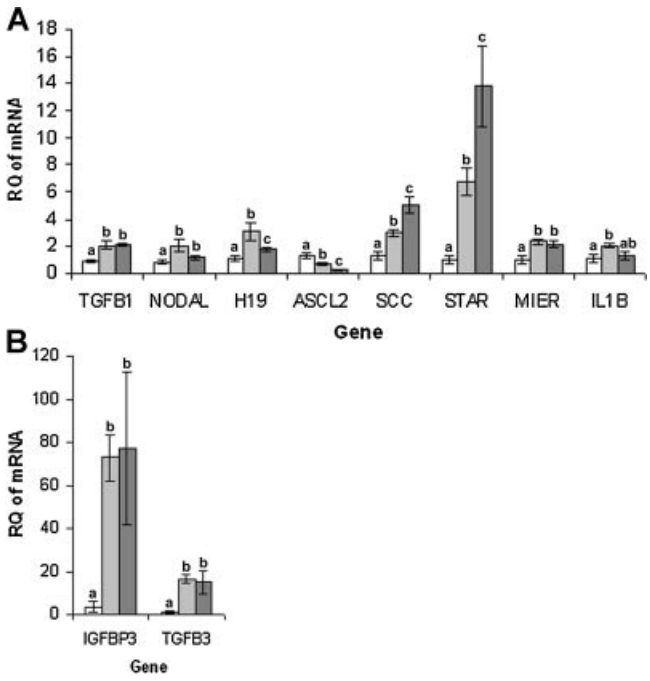

Figure 4. Confirmation of differential expression of trophoblast regulating genes. Ten genes were selected based on their known regulation of trophoblast characteristics for validation of their differential expression by real-time PCR. The relative quantity (RQ) was normalized to β-actin. Due to the difference in the RQ value for some of the transcripts analyzed, two distinct bar graphs (**A** and **B**) were generated to better depict the differences between CD and DD concepti for the ten genes examined. The RQ values shown by the various bars are the mean ± standard error; white bars (CD concepti), light gray (DD concepti) and dark grey (filamentous concepti). Means without a letter in common differ ($P < 0.05$).

DISCUSSION

In this study, in-depth genomic analysis demonstrates for the first time, the impact the uterine milieu, which includes more advanced concepti, has on the conceptus' transcriptome, thus potentially, its physiological state during the early phase elongation. The results suggest that the morphology of the extra-embryonic membranes is secondary to the environment. Clearly, the more developmentally advanced a conceptus is, a different set of responses is elicited from the maternal endometrium. Prior support for the influence of the uterine environment on the developmental potential of pig concepti during elongation has been derived from either a direct manipulation of E2 levels within the uterine tract (Blair et al., 1991; Geisert et al., 1991) or the transfer of embryos to recipients with a less or more developed uterine tract prior to the elongation stage (Pope et al., 1986; Wilde et al., 1988). In addition, our correlation/cluster analyses results demonstrated that the transcriptome profile of distinct ovoid concepti was sufficient to determine the population of origin. However, more importantly, could information be gleaned with respect to the conceptus' developmental status? Analyses of classical pathways and networks by IPA to pinpoint a specific factor(s) that could have a prominent role in physiological processes highlighted TGFβ1 within the most significant network. Though the interactions were indirect, TGFβ1 exhibited a central role in the modulation of genes involved in the regulation of a myriad of biological processes.

With elongation, dramatic gross developmental changes occur within the extra-embryonic membrane, a tissue that consists of two-cell layers, the endoderm and polarized trophectoderm. In the early blastocyst (∼gd6), the cell number of the extra-embryonic membrane is about threefold that of the ICM; this disparity is greatly amplified by gd11 and 12 (Degrelle et al., 2009). Additionally, the endoderm to trophoblast ratio is ∼1:5 (Degrelle et al., 2009), which makes the cells of the trophectoderm the most prominent cell type during elongation. The expression level of more than 200 transcripts was found to be significantly altered in ovoid concepti surrounded by the more advanced concepti. One may consider that many of the transcripts detected likely come from the more abundant trophectodermal cells but one cannot negate that some of the transcripts detected may be from the ED. For example NODAL, it has been shown to be important for formation of the mesoderm, primitive streak and embryonic asymmetry (Iannaccone et al., 1992; Hassoun et al., 2009). However, the discussion with respect to the differentially expressed transcripts identified will concentrate on their potential role in the trophectoderm.

The TGFβ protein superfamily growth factors have cytokine-like properties and are involved in several facets

TABLE 2. Comparison of Fold Changes Between Distinct Conceptus Categories

Transcript	Microarray fold change DD to CD	Real-time PCR fold change DD to CD	Real-time PCR fold change DD to FIL	Real-time PCR fold change CD to FIL
TGFβ1	2.55	2.5	—	2.6
TGFβ3	55.76	13.6	—	12.0
NODAL	2.49	2.6	1.8	1.5
IL1β	—	1.9	1.6	—
STAR	6.9	7.0	−2.0	14.4
CYP11A	2.04	2.3	−1.7	3.9
H19	3.06	1.9	1.7	1.6
ASCL2	−2.86	−2.1	2.2	−4.6
IGFBP3	109.39	21.0	—	2.1
MIER	2.53	2.4	—	1.5

Conceptus stage: DD, developmentally delayed ovoid; CD, control ovoid; FIL, filamentous.

of cellular growth and differentiation within a variety of tissues (Jones et al., 2006). The physiological role of each unique TGFβ protein is typically distinct and the activity of the protein is dependent upon its microenvironment, that is, cell type, status of cell differentiation or growth, and presence of interacting factors (Jones et al., 2006). Cross-talk between different TGFβ family member pathways (TGFβ1, TGFβ3, NODAL) or other developmental factors such as, IL1β, are also vital for conceptus/placental development (Iannaccone et al., 1992; Karmakar and Das, 2002; Munir et al., 2006; Ng et al., 2006).

In the porcine reproductive tract, the mRNA and protein of TGFβs are synthesized in the endometrial and conceptus tissues and levels, including active protein forms, increase with progression through elongation and implantation (Gupta et al., 1998a,b). The up-regulation of TGFβs is coincident with peaks in E2 synthesis by the conceptus, a factor known to induce the expression of TGFβs (Rama et al., 2004). The presence of TGFβs and their activation complexes at the maternal:fetal interface and the ability of TGFβ to positively regulate molecules associated with adhesion, such as fibronectin, integrin, or osteopontin, suggest that TGFβ may play an important role in embryo attachment (Jaeger et al., 2005; Massuto et al., 2010). In species with more invasive placentas, TGFβs modulate trophoblast migration and invasion into the endometrium and decidulization of the stroma (Jones et al., 2006). Thus, irrespective of the form of placentation, TGFβs play an important role in implantation.

Alterations in the expression or regulation of TGFβ family members have been associated with placental pathology. For example, in human pre-eclampsia or intrauterine growth retardation, TGFβ3 is preferentially up-regulated and trophoblast invasion of the endometrium is diminished; restoration of TGFβ3 to the appropriate levels re-establishes their invasiveness (Caniggia et al., 1999). Over-expression of NODAL, which can share receptors with TGFβ1 or lie downstream in the TGFβ1 cascade, leads to increased apoptosis in cultured first trimester transformed human trophoblasts (Munir et al., 2004), whereas the inhibition of NODAL expression in mid-gestation murine trophoblasts alters their differentiation and promotes the abnormal accumulation of a trophoblast subtype (Ma et al., 2001); both scenarios compromise placentation. Thus, tight control of TGFβ family members is important for proper placentation.

In DD concepti, the transcript level of TGFβ1, TGFβ3, and NODAL was significantly increased. The mRNA level of each gene in the DD conceptus approached that found in the filamentous conceptus; however, for TGFβ3 the increase was 13-fold above the CD conceptus level whereas the difference was approximately 2.5-fold for TGFβ1 and NODAL. Additionally, NODAL was 82% greater in the DD conceptus than filamentous, potentially creating an imbalance between TGFβ family members. By gd12, concepti have migrated to the location of the uterus where they will implant. Beyond gd12, proliferation of the trophoblast cells is prominent as the trophectoderm initiates permanent contact with the endometrial tissue of the uterus. Though the pig has a non-invasive epitheliochorial placenta in which only the trophoblast villi invade and interdigitate with endometrial tissue during implantation (Geisert and Malayer, 2000), outside the uterine lumen, porcine trophoblasts exhibit invasive characteristics similar to their human counterpart (Samuel and Perry, 1972). Therefore, disparity between the developmental stage of the DD concepti and the expression of these TGFβ family members could disrupt cell migration processes and the interaction of trophoblast villi with the endometrium that together compromise implantation, subsequent placentation and overall development of the conceptus.

Network analysis indicated TGFβ1 was potentially upstream of 24 differentially regulated factors, including TGFβ3, that could regulate a number of distinct biological activities in the embryo. Of particular interest, considering the importance of E2, were STAR, the ultimate regulator of steriodogenesis, and CYP11A1, the rate-limiting enzyme in steroid production. Two other steroid-related transcripts detected were dehydrogenase type 1 (HSD11B1) and MIER1. The HSD11B1 protein catalyzes the interconversion of physiologically active cortisol to the inert metabolite, cortisone (Michael and Papageorghiou, 2008). Glucocorticoids, unlike progesterone, also seem to regulate trophoblast differentiation and association with the endometrium (Malassiné and Cronier, 2002). The MIER1 protein in non-trophoblastic epithelial cells works at the receptor level where it can sequester E2 receptor α and limit E2 activity (McCarthy et al., 2008). In the human trophoblast, TGFβ1 can regulate E2 synthesis and initiate its terminal differentiation (Croniera et al., 1999; Rama et al., 2004). As alluded to earlier, E2 is vital for the maternal recognition of pregnancy in swine and is also a strong mitogen that regulates the growth and differentiation of pig trophoblasts (Ka et al., 2001). Both E2 receptor α and β are thought to modulate trophoblast differentiation or proliferation in humans (Bukovsky et al., 2003) and presence of the receptor in the porcine trophectoderm during elongation indicates a role in pig as well (Ka et al., 2001; Kowalski et al., 2002).

The STAR, CYP11A1, MIER1, and HSD11B1 transcripts were increased in DD concepti and the expression profile of the first three was confirmed; HSD11B1 was not evaluated by real-time PCR. Both STAR and CYP11A1 exhibited an intermediate range between the CD and filamentous conceptus whereas the MIER level was similar to the filamentous conceptus. Considering TGFβ1 can up-regulate E2 synthesis in the trophoblast, it was not surprising that potential regulators of E2 synthesis, STAR and CYP11A1, were downstream of TGFβ1 in the network analysis. If the DD concepti are truly compromised it could be potentially through several routes involving E2. First, an increase in E2 could prematurely drive the differentiation of trophoblasts to subtypes not yet defined in pig. Second, the endometrial microenvironment, in response to an increase in E2 secretion by the conceptus and surrounding tubular/filamentous concepti, may not be in synchrony with the conceptus' developmental state. And third, a similar increase in MIER protein may minimize the autocrine effect of E2 on the maturation of conceptus' trophoblasts via MIER sequestration of the α receptor.

Transcript expression changes of two genes known to be imprinted in the placenta, ASCL2 (Guillemot et al., 1995) and

H19 (Jinno et al., 1995), and a third, IGFB3, that controls the bioavailability of the imprinted gene IGF2 (Chao and D'Amore, 2008) were also detected in the DD concepti. Though it was recently shown that ASCL2 is not imprinted in the pig placenta (Bischoff et al., 2009), the requirement of ASCL2 for the development of trophoblast progenitors (Guillemot et al., 1995), the rapid proliferation of trophoblasts (Arnold et al., 2006), and efficient placental development and fetal growth (Guillemot et al., 1995; Arima et al., 1997; Guo et al., 2008) and its differential expression between CD ovoid and filamentous stages in porcine concepti, suggest ASCL2 is still likely important for development of the primordial placental tissue in the pig. In this study, H19 and ASCL2 transcript expression was increased and decreased, respectively, in the DD conceptus compared to CD. The mRNA level of H19 and ASCL2 was particularly interesting because it was significantly different from the filamentous concepti as well; H19 and ASCL2 were more highly expressed in the DD than the filamentous concepti. Though a change in IGF2 expression was not detected, there was a 21 -fold increase in IGBP3. Provided the IGFBP3 protein expression mimics its mRNA expression, this could provide a mechanism to reduce the bioavailability of IGF2. The induction of IGFBP3 expression has been shown to be regulated by TGFβ1 in human trophoblasts (Feng et al., 2005) and the inhibition of human trophoblast proliferation via IGFBP3 can occur in an IGF-independent manner through TGFβ receptor signaling (Forbes et al., 2010). Despite the obvious difference in the invasive properties of the mouse or human trophoblast compared to that of porcine, dramatic remodeling of the extra-embryonic membranes does require proliferation during elongation. An elevated level of IGFBP3 in the DD conceptus without a concomitant change in IGF2 suggest it might be important to look at the role IGFBP3 in the modulation of porcine extra-embryonic growth via TGFβ at this time of development. With respect to H19 and ASCL2, an impact of TGFβ1 on their expression has not been described yet. Overall, the expression directionality of ASCL2 and H19 and potential suppressed IGF2 activity and their association with placental pathology infer that the development of the extra-embryonic membranes may be at risk in DD concepti.

Simultaneous with blastocyst elongation in the pig is modification of the endometrium. Changes within the endometrial stroma to enable implantation of the conceptus occur during a restricted window of uterine receptivity. In addition to the importance of ovarian hormones for priming of the endometrium in anticipation of implantation, IL1β synthesis and secretion by the embryo's trophectoderm promotes decidualization of the endometrial stroma in species with invasive placentas (Grümmer et al., 2004; Staun-Ram and Shalev, 2005; Ng et al., 2006). Concomitantly, the process is antagonized by TGFβ1 inhibition of protease involved in degradation of the endometrium (Jones et al., 2006). Whether IL1β is involved in the interdigitation of the trophoblastic microvilli with the stroma or immune modulation (Ross et al., 2003) of the endometrium remains to be determined in swine. Differential expression of IL1β mRNA, though not detected by microarray, has been shown in the porcine embryo and is one of the most abundant transcripts present during elongation (Ross et al., 2003; Blomberg et al., 2006), so it was examined due to its importance in implantation. Expression of IL1β mRNA in DD concepti surpassed the level of CD and filamentous concepti. Interestingly, in species with hemochorial placentas IL1β and TGFβ pathways intersect and have opposing effects with respect to implantation; IL1β promotes invasiveness and TGFβ1 antagonizes that process through the inhibition of proteases (Staun-Ram and Shalev, 2005; Ng et al., 2006). However, without knowing IL1β's physiological role or its interaction, if any, with TGFβ1 in the pig endometrium it is difficult to ascertain how the altered expression of these two genes may affect synchrony between the endometrium and conceptus in the pig.

In conclusion, in-depth profiling of morphologically similar CD and DD concepti demonstrated that the mRNA expression of multiple factors that may regulate trophoblast characteristics and/or modifications of the maternal endometrium, in particular TGFβ1 and associated genes, were altered in the DD conceptus. Though the mRNA expression profile of candidate genes was more similar between DD concepti and filamentous concepti, the existence of differences between some genes imply that the DD and filamentous conceptus are also distinct. Our data suggested that morphology alone should not be used for porcine embryo classification; developmentally delayed concepti and control counterparts have distinct transcriptomes. It may be of interest to examine further whether these changes could be indicative of progression in the development of the conceptus or abnormal expression by a less mature embryo in a more mature environment. That said, it is also important to point out that even though CD concepti appeared to advance normally, they were derived from superovulated oocytes and DNA and transcriptome modifications have been associated with superovulation in some species (Fauque et al., 2007; Mundim et al., 2009). Interestingly, recent studies indicate that superovulation may not affect the oocyte itself (Anckaert et al., 2009). Functional studies with comparisons of gene expression with non-super ovulated DD concepti or their transfer to synchronized recipients, as have been done with earlier embryos, will be vital to unravel the influence of hormonal treatment and uterine environment on developmental competence of the porcine conceptus. However, the difficulty to attain DD concepti during gd11–11.5 and the conceptus' fragility make this type of endeavor extremely difficult. Furthermore, an examination of the expression profiles of the genes described herein within actual models of placental pathology in the pig, such as intrauterine growth retardation, may also provide some insight into their role in the placenta critical stages of post-implantation.

MATERIALS AND METHODS

Animals

Hybrid gilts 6 months of age or older with a normal estrous cycle and weighing at least 100 kg were superovulated and mated using artificial insemination for in vivo conceptus production (Blomberg et al., 2005). Animals were maintained in the same facilities under identical conditions and food rations. Ovoid concepti undergoing elongation were collected within a 3-hr window at gd11–11.5,

measured, and grouped according to the morphology of the conceptus population from which they were collected. Individual ovoid embryos 7–8 mm were classified as CD if they originated from a homogenous population of ovoid concepti or DD if they came from a heterogeneous population of concepti in which the majority were advanced tubular or filamentous concepti (see Fig. 1 for different conceptus morphologies). Embryos were collected on four distinct days for the CD and DD concepti over a period of 1.5 years; on 2 of the days both CD and DD were collected from distinct animals. It is important to note that the heterogeneous gd11 embryo populations could be obtained from animals euthanized at any time during that 3 hr window; that is, they were not preferentially derived from animals closer to the gd11.5 timepoint. Filamentous concepti from homogenous populations, that is, filamentous morphology but not all the same size with respect to length, were collected at gd12 as clumps (~5 concepti/pool by determination of ED) to minimize handling and avoid potential tissue tears (Blomberg et al., 2005). All animal protocols were approved by the Beltsville Area Animal Care and Use Committee and meet the USDA and NIH guidelines for the care and use of animals.

Total RNA preparation

For microarray analysis, gd11 CD ovoid concepti used were obtained from three distinct gilts on 3 different days and the same was done for DD ovoid concepti. An additional two gd11 CD and three DD concepti from distinct animals were used for confirmation by gene expression by real-time PCR downstream. Embryos were collected from flushes with a similar range of embryos per tract: CD group—9, 12,15, 23, and 27; DD group—10, 16, 17, 18, 21, and 30. Likewise filamentous embryos were collected from three distinct gilts on different days. Total RNA from the individual CD and DD ovoid concepti and unique filamentous concepti pools was isolated using the RNeasy microKit (Qiagen, Valencia, CA). An on-column DNase I digest of each conceptus sample was performed according to the manufacturer's instructions to remove genomic DNA contaminants. Total RNA integrity and quantification were determined with the Agilent RNA 6000 Nano LabChip Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and the quality was very good and consistent between samples.

mRNA Amplification and cRNA labeling

The mRNA from the four gd11 CD and four DD ovoid concepti was used to generate Cy3 and Cy5 labeled cRNA with the Agilent Low RNA Input Linear Amplification Kit PLUS, Two-Color Kit (Agilent, Santa Clara, CA) for microarray analysis. The cRNA was prepared according to the manufacturer's instructions for two-color labeling. The Spike A Mix for cyanine 3 (Cy3) and Spike B Mix for cyanine 5 (Cy5) labeling was serially diluted 1:20, 1:40, and 1:4. Duplicate cDNA synthesis reactions that contained either 2 μl of diluted (1:4) Spike A Mix (for Cy3 labeling reaction) or Spike B Mix (for Cy5 labeling reaction) were set up for each conceptus sample with 1 μg total RNA, 1.2 μl T7 promoter primer, 1× first-strand buffer (kit), 10 mM dithiothreitol (DTT), 0.5 mM dNTP, 1 μl MMLV-reverse transcriptase, and 0.5 μl RNase. Samples were incubated at 40°C for 2 hr followed by a 15-min incubation at 65°C. The cRNA synthesis/amplification and incorporation of cyanine label was performed utilizing cDNA synthesis products in an 80 μl reaction mix containing 1× transcription buffer (kit), 10 mM DTT, 8 μl of the NTP mix, 4% polyethylene glycol, 0.5 μl RNaseOUT, 0.6 μl inorganic pyrophosphate, 0.8 μl T7 RNA polymerase, and 2.4 μl of Cy3-CTP or Cy5-CTP. The in vitro transcription reaction was carried out for 2 hr at 40°C and the amplified cRNA was purified using an RNeasy mini column according to the procedure described in Low RNA Input Linear Amplification Kit. Quantification of cRNA and cyanine dye incorporation was determined with the NanoDrop ND-1000 UV–Vis spectrophotometer using the Microarray Measurement tab. All cRNA probes used for microarray analysis had a specific activity >0.8 pmol Cy3 or Cy5/μg cRNA.

Agilent Custom Porcine E-Array

A custom porcine microarray was designed by E-array (Agilent Technologies) in a 4 K × 44 K array format utilizing porcine transcript information in the NCBI Pig Unigene database (http://www.ncbi.nlm.nih.gov/UniGene/UGOrg.cgi?TAXID=9823). Microarray probes were hybridized with labeled target CD or DD cRNA as recommended by Low RNA Input Linear Amplification Kit instructions. For each array, 825 ng of the Cy3 and 825 Cy5 of labeled cRNA generated from the total RNA of the same conceptus were mixed together. The dual labeled cRNA was fragmented in the presence of 11 μl 2× Blocking Agent and 1× fragmentation buffer in a total volume of 55 μl for 30 min at 60°C. Fifty-five microliters of 2× GEx Hybridization Buffer HI-RPM was added to each cRNA sample to stop the fragmentation process and samples were place on ice. The fragmented Cy3-labeled cRNA and Cy5-labeled cRNA probe mixture of each distinct CD or DD ovoid conceptus were mixed together in equimolar amounts to hybridize to targets on the array for 17 hr at 65°C in the Agilent hybridization oven with a rotator setting of 10 rpm. Microarray slides containing the arrays were disassembled submerged in Gene Expression wash buffer 1 at room temperature (rt) and placed in a slide rack. Subsequent washes of slides were performed in GE wash buffer 1 for 1 min at rt, pre-warmed 37°C GE wash buffer 2 for 1 min, and 100% acetonitrile for 1 min. The Cy5 dye was stabilized by placing in the ozone scavenging Agilent Stabilization and Drying solution for 30 sec. Slides were scanned on the Agilent Microarray Scanner (Agilent) using the Gene Expression version v.5 settings required for 4× 44 K dual color arrays to capture intensities for each target/probe. All the microarray raw data has been deposited in the National Center for Biotechnology Information Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE18472.

Microarray Data Analysis

To increase stringency of analysis, data from only targets labeled by both probes (i.e., yellow spots) were utilized for further analyses; if the target was labeled with only the Cy3 or Cy5 probe (i.e., red or green spots, respectively) it was excluded. Background-adjusted, normalized intensity values from the gProcessedSignal (Cy5) and rProcessedSignal (Cy3) were log2 transformed and analyzed separately utilizing the significance analysis of microarrays (SAM) method version 3.02 (http://www-stat.stanford.edu/-tibs/clickwrap/sam/academic/; Storey and Tibshirani, 2003) with a two-class unpaired design and an FDR ≤ 5% as described previously in Li et al. (2007). QC analyses of the arrays, that is, the correlation coefficient, hierarchical clustering, and PCs of the LS means, were performed with the JMP Genomics software v. 3.2 (SAS, Cary, SC) using intensity log2 transformed Cy5 and Cy3 values to examine the relationship between the distinct microarray runs and potential segregation of expression profiles according to labeling fluorophore (Cy3 or Cy5) and origin of sample, that is, CD or DD.

Real-Time RT-PCR

Total RNA from six distinct gd11 CD concepti and seven distinct DD concepti, which included those used for microarray, as well as three individual pools of gd12 filamentous concepti was used as template for real-time RT-PCR. Gene-specific primers (Supplementary Table 2) were designed from porcine-specific sequences for TGFβ1, TGFβ3), IGFBP3, IL1β, ASCL2, H19), CYP11A, STAR (longest transcript variant, see Blomberg and Zuelke 2005), NODAL, and MIER1 obtained from NCBI's GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez) or the Gene Index

Project at Harvard's Computational Biology and Functional Genomics Laboratory (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pig) using Primer Express (Applied Biosystems, Foster City, CA). Reverse transcription, real-time PCR, and statistical analysis by ANOVA were performed as described previously (Blomberg et al., 2005); the only modifications were a dilution of the reverse transcription product to 1.5 ng RNA input equivalents/μl and the use of 5 μl/PCR rxn. Beta actin was statistically evaluated for its use as the endogenous control; the mean for each sample across six distinct PCR runs was determined and analyzed by ANOVA to demonstrate that ACTB was not differentially expressed between CD and DD embryos. All the results were expressed as the relative quantity (RQ) of the transcript of interest to the endogenous control ACTB (Blomberg et al., 2005).

Bioinformatic Anlaysis: Annotation and Functional Characterization

Official gene name symbols in ENTREZ gene (NCBI, http://www.ncbi.nlm.nih.gov/sites/entrez/?db=gene) were uploaded into the GeneCoDis 2.0 web-based tool (http://genecodis.dacya.ucm.es) and GO annotations of biological processes (BP) and cellular component (CC) were determined. The GOSlim feature utilized is an abbreviated version of the in-depth GO content and it provides a broad overview of the ontological content. The search focused on BPs and molecular functions; the default parameters were as follows: no *P*-value correction, hypergeometric statistical test, and a minimum of three genes within the specified process or function. The official gene names and the expression fold difference between DD and ND were also uploaded into the IPA version 7.5 software (Ingenuity® Systems, Redwood City, CA; www.ingenuity.com) to identify putative networks and well-defined canonical pathways represent by the population of input genes. The network analysis depicts graphically the molecular relationship (direct or indirect interaction) between protein:protein, protein:chemicals, and protein:nucleic acid based on literature and, the potential biological outcome of their interaction.

ACKNOWLEDGMENTS

The authors would like to thank to Russ Lange, Clayton Wieben, Jim Piatt, and Dale Buschling for their help with animal management; Dr. Neil Talbot for providing technical assistance with animal management through Paul Graninger; Dr. Jeremy Miles, Dr. H. David Guthrie, and Dr. Julie Long for review of the manuscript. This study was supported by USDA ARS CRIS Project No. 1265-31000-094.

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